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MACROPHAGE POPULATION DYNAMICS WITHIN FETAL MOUSE FIBROBLAST CULTURES DERIVED FROM C57BL/6, CD-1, CF-1 MICE AND INTERLEUKIN-6 AND GRANULOCYTE COLONY STIMULATING FACTOR KNOCKOUT MICE

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SUMMARY

In vitro models of macrophage growth, differentiation, and function are needed to facilitate the study of their biology as important immune facilitator cells and as frequent targets of bacterial and viral infection. A simple method for the selective expansion and continuous culture of mouse macrophages from primary explant cultures of mouse embryonic tissue is described. Culture in Dulbecco modified Eagle medium (DMEM) low-glucose (1 g/L) formulation (DMEM/L) inhibited fibroblast growth. In contrast, macrophages continued to proliferate in the presence of DMEM/L when in contact with the fibroblasts. Alternating growth in high-glucose DMEM with DMEM/L produced a 1.16- to 2.1-fold increase (depending on mouse strain) in the percentage of macrophages within the cell culture in comparison with culturing in DMEM with high glucose exclusively. Macrophage yields of over 1 million cells/T12.5 flask were achieved by passages 3–4, and, thereafter, declined over the next 5–10 passages. The peak percentage of macrophages within a culture varied depending on the strain of mouse (C57BL/6, CD-1, and CF-1 and two knockout C57BL/6 strains deficient in either interleukin-6 [IL-6] or granulocyte colony stimulating factor [GCSF]). The GCSF (−/−)-derived cultures had the lowest peak macrophage content (30%) and CD-1 the highest content (64.9%). The IL-6 (−/−) and CD-1 cultures appeared to spontaneously transform to create cell lines (IL6MAC and CD1MAC, respectively) that were composed of 50–75% macrophages. The macrophages were phagocytic and were positive for CD14, acetylated low-density lipoprotein receptors, and F4-80 antigen. Light and electron microscopy showed that the cultured macrophages had in vivo–like morphological features, and they could be plated to high purity by differential attachment to petri dishes in serum-free medium.

Key words: cell culture; macrophage; mouse.

INTRODUCTION

Macrophages are important cells for innate and adaptive immunity and are also critical for the phagocytosis of dead or dying cells and other debris such as modified proteins (Unanue and Allen, 1987; Auger and Ross, 1992). Partly because of these functions, macrophages are also a primary target cell for many viruses and bacteria that invade the body (McCullough et al., 1993). To facilitate the study of these and other macrophage characteristics, culture methods allowing the in vitro growth and experimental manipulation of macrophages have long been studied.

For many yr, various transformed macrophage cell lines have been developed and they have been effectively used in investigating the cell biology of these very important immunocytes (Walker, 1994). However, nontransformed macrophages are difficult to culture continuously and are often viewed as terminally differentiated cells (Freshney, 1994). As a result, studies requiring normal nontransformed monocytes or macrophages most often use cells harvested from blood, peritoneal exudates, or the lungs. From all these in vivo sources, considerable purification steps are necessary to obtain relatively pure populations of monocytes or macrophages for experimentation (Adams, 1979). Thus, a simple method for the routine continuous culture of normal mouse macrophages would be of value.

We have previously shown that fetal tissue–derived or blood-derived monocyte or macrophages of the pig can be continuously cultured on feeder layers made from the STO mouse fibroblast cell line (Talbot and Paape, 1996; Talbot et al., 1998). This study describes a similar simple method for the continuous culture of normal mouse macrophages from embryonic tissues. The method is based on the propagation and differential growth of macrophages that are interspersed among the fibroblasts (or fibroblast-like cells) that typically make up the bulk of a primary explant cell culture outgrowth. The in vitro population dynamics of macrophages in mouse fibroblast cultures derived from five strains of mice were assayed. Three strains of mice, C57BL/6, CD-1, and CF-1, were examined to test whether differences existed in macrophage proliferation among mouse fibroblast cultures that have been used in the isolation or propagation of mouse or human embryonic stem cell lines. Fibroblast cultures from two knockout strains of mice with immune im-

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pairment, interleukin-6 (IL-6) −/− (Kopf et al., 1994) and granulocyte colony stimulating factor (GCSF) −/− (Lieschke et al., 1994), were assessed for comparison to see whether in vivo deficiencies in macrophage number or function would influence macrophage proliferation in the culture system.

Materials and Methods

Animals. C57BL/6 mice, GCSF-deficient mice (B6;129P2-*Csf3*^{tm1Ard}; Lieschke et al., 1994), and IL-6–deficient mice (B6.129S2-*Il6*^{tm1Kopf}; Kopf et al., 1994) were purchased from the Jackson Laboratory, Bar Harbor, Maine. The mice were housed and bred (and fetuses were collected) in accordance with a protocol approved by the Beltsville Area Animal Care and Use Committee. Pregnancies were produced by natural matings and fetuses were collected at approximately 16–18 d postcoitus.

Cell culture. All cells were grown in 25-cm^2 tissue culture flasks (T25 flask, Greiner, Frickenhausen, Germany) or 12.5-cm^2 tissue culture flasks (T12.5 flask, Falcon, Becton Dickinson, Lincoln Park, NJ) and cell culture reagents including fetal bovine serum (FBS), Dulbecco phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++}, trypsin–ethylenediamine-tetraacetic acid (EDTA) (0.05% trypsin, 0.53 m*M* EDTA), media and media supplements were obtained from InVitrogen (GIBCO), Gaithersburg, Maryland. Primary cultures of mouse embryonic fibroblast–macrophages (MEF/Mac) were initiated by explant culture of day −16 to −18 gestation fetal mice after removal of viscera (Freshney, 1994). Finely minced embryonic mouse tissues were plated in T25 flasks in 2 ml of Dulbecco modified Eagle medium (DMEM) with 4.5 g/L glucose (high glucose) supplemented with 10% FBS, 2 m*M* glutamine, and 50 U/ml penicillin–streptomycin (10% DMEM/H). After attachment and initial outgrowth (~96 h), 4 ml of additional 10% DMEM/H was added to each primary culture flask. Secondary cultures were produced by washing the primary cultures twice with PBS and once with trypsin–EDTA solution. The released cells were resuspended in 10% DMEM/H and replated in T25 flasks at a 1:3 split ratio. The MEF/Mac cultures were each frozen down the d after passage in 1 ml aliquots of 92% FBS–8% dimethylsulfoxide by quick freezing on powdered dry ice and storage in liquid nitrogen vapor. The C57BL/6 cultures were frozen at passage 1 (P1), the GCSF (−/−) cultures at passage 2 (P2), and the IL-6 (−/−) cultures at P1. CD-1 primary embryonic fibroblast cultures prepared in a similar manner from day 14 fetal mice and frozen down at P2 were obtained from StemCell Technologies, Inc., Vancouver, British Columbia, Canada. Primary CF-1 embryonic fibroblast cultures started from day 13 fetal mice and frozen at P1 were kindly provided by Dr. James Thomson, University of Wisconsin, Madison, Wisconsin.

CD14 receptor assay. CD14 is a single chain membrane glycoprotein with a molecular weight of 55 kDa that is found principally on the surface of monocytes and tissue macrophages (Wright et al., 1990). The presence of CD14 is commonly used as a marker of these cells in flow cytometry studies. The MEF/Mac cells were washed once with PBS to remove medium. Cells were adjusted to 1×10^8 cells/ml with PBS. Twenty-five microliters of each cell suspension was added to each of two plastic vials. Two-hundred microliters of a 1:10 dilution of fluorescein isothiocyanate (FITC)–conjugated rat anti-mouse CD14 monoclonal antibody (rmC5-3; PharMingen, San Diego, CA) was added and allowed to incubate for 30 min at 4° C. Cells were washed three times with PBS. An Epics Profile flow cytometer (Coulter Electronics, Hialeah, FL), equipped with an air-cooled argon ion laser, was used to determine the percentage of fluorescent cells and the log mean fluorescent channel, an indicator of receptor density. To check for lymphocytes, the MEF/Mac cells were similarly processed with 200 μl of a 1:10 dilution of FITC-conjugated rat anti-mouse CD25 (IL-2 receptor α-chain, p55) monoclonal antibody that is reactive with B- and T lymphocytes.

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate–acetylated low-density lipoprotein uptake and cell growth assay. Macrophages were shown to internalize acetylated low-density lipoprotein (LDL) by specific receptor-mediated processes (Goldstein et al., 1979). The MEF/Mac cultures were exposed to 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate–acetylated LDL (DiI-Ac-LDL) at 10 μg/ml in 10% DMEM/H for 4 h at 37° C according to the manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA). Quantitative removal of the cells from T12.5 flasks was achieved with two PBS rinses followed by the addition of 0.5 ml trypsin–EDTA (0.25% trypsin for CD-1 and CF-1 cell cultures), and the cells were resuspended in 10 ml of 10% DMEM/H medium. Counts of total cells and DiI-Ac-LDL(+) cells were performed with a hemocytometer. In almost all cases, 16 (but at least eight) large squares of the hemocytometer were counted for each cell culture sample. Cells that had taken up DiI-Ac-LDL were observed using a rhodamine excitation–emission filter set on an Olympus IMT-2 inverted microscope fitted with an Olympus IMT-2 reflected light fluorescence attachment (Opelco, Washington, DC).

Phagocytosis assay. Phagocytosis was measured using a modified flow cytometric method (Saad and Hageltorn, 1985) with modifications as described previously (Talbot and Paape, 1996; Talbot et al., 1998) and reaction aliquots taken from MEF/Mac cells at 1×10^8 cells/ml and from *Staphylococcus aureus* at 1×10^{10} cells/ml.

Transmission electron microscopy. Transmission electron microscopy (TEM) sample preparation and photomicroscopy were conducted with the assistance of JFE Enterprises, Brookeville, Maryland, as described previously (Talbot et al., 1998, 2000). Ultrastructural analysis was performed in situ on one T25 flask culture that was 7 d postpassage, which had been refed at 3 d postpassage with DMEM containing 1 g/L glucose (low glucose) and supplemented with 10% FBS, 2 m*M* glutamine, and 50 U/ml penicillin–streptomycin (10% DMEM/L), and on similarly cultured MEF/Mac cells that had been released from the plastic substrate by trypsin–EDTA, used in the *S. aureus* phagocytosis assay, and centrifuged into a pellet.

Immunocytochemistry. The F4/80 antibody defines a murine macrophage–restricted cell surface glycoprotein (McKnight et al., 1996). Immunofluorescent labeling of C57BL/6 MEF/Mac cultures with rat monoclonal F4/80 antibody (ab6640; Abcam, Ltd., Cambridge, UK) was performed as described previously (Talbot et al., 2003). Cell cultures at P5 were grown on Permanox chamber slides (Nalge Nunc International, Naperville, IL) using 10% DMEM/H for 3 d of culture followed by 10% DMEM/L for 4 d. Cells were fixed for 30 min in 4% methanol-free formaldehyde in PBS. Alexa 488–labeled goat anti-rat secondary antibody (Molecular Probes, Eugene, OR) was used to detect binding of the primary F4/80 antibody. The actin cytoskeleton was stained with Alexa fluor 594 phalloidin (Molecular Probes) at 2 U/ml (66 n*M*). Cell nuclei were counterstained with 2 μg/ml bisbenzimide (Hoechst 33342; Molecular Probes) and the specimens were mounted in Vectashield (Vector Labs., Burlingame, CA).

Results

Primary explant cultures of mouse embryonic fibroblasts (MEFs) were initiated in 10% DMEM/H by standard methods in T25 tissue culture flasks (Freshney, 1994). The explant cultures of mouse embryonic tissue presumably contain macrophages because these cells are resident in most, if not all, tissues of the embryo (Naito et al., 1996), and they have been propagated from explant cultures in other species such as the pig (Talbot and Paape, 1996). Mouse macrophages interspersed among the MEFs were first tentatively identified by their characteristic morphology (Naum, 1975; Bennett et al., 1993; Talbot and Paape, 1996). Using phase-contrast microscopy, the presumptive macrophages were phase-contrast dark cells that either occurred as semiattached, round, refractile cells or as closely adherent, flat, amoeboid cells, often with several extended pseudopods and prominent intracellular vacuoles (Fig. 1*A* and *C*). These same cells were also positive for the uptake of DiI-Ac-LDL (Fig. 1*B*), a characteristic of macrophages by virtue of their having receptors for acetylated LDL (Goldstein et al., 1979). Using microscopic evaluation, macrophages were seen to be lost from the C57BL/6 explant cultures with time and continued passage of the cells in 10% DMEM/H. Hypothetically, this occurred as a result of faster growth by the fibroblast population in the culture. To test this hypothesis, a simple method for slowing the growth of the fibroblasts was sought. Lowering the amount of glucose in the medium was tested for its effects on the MEF/Mac cultures by switching the cultures to 10% DMEM/L. It was observed that the MEF were inhibited in their growth in the 10% DMEM/L after 3 d. With continued culture in 10% DMEM/L, microscopic evaluation indicated that the replication of the macrophages was not inhibited by the

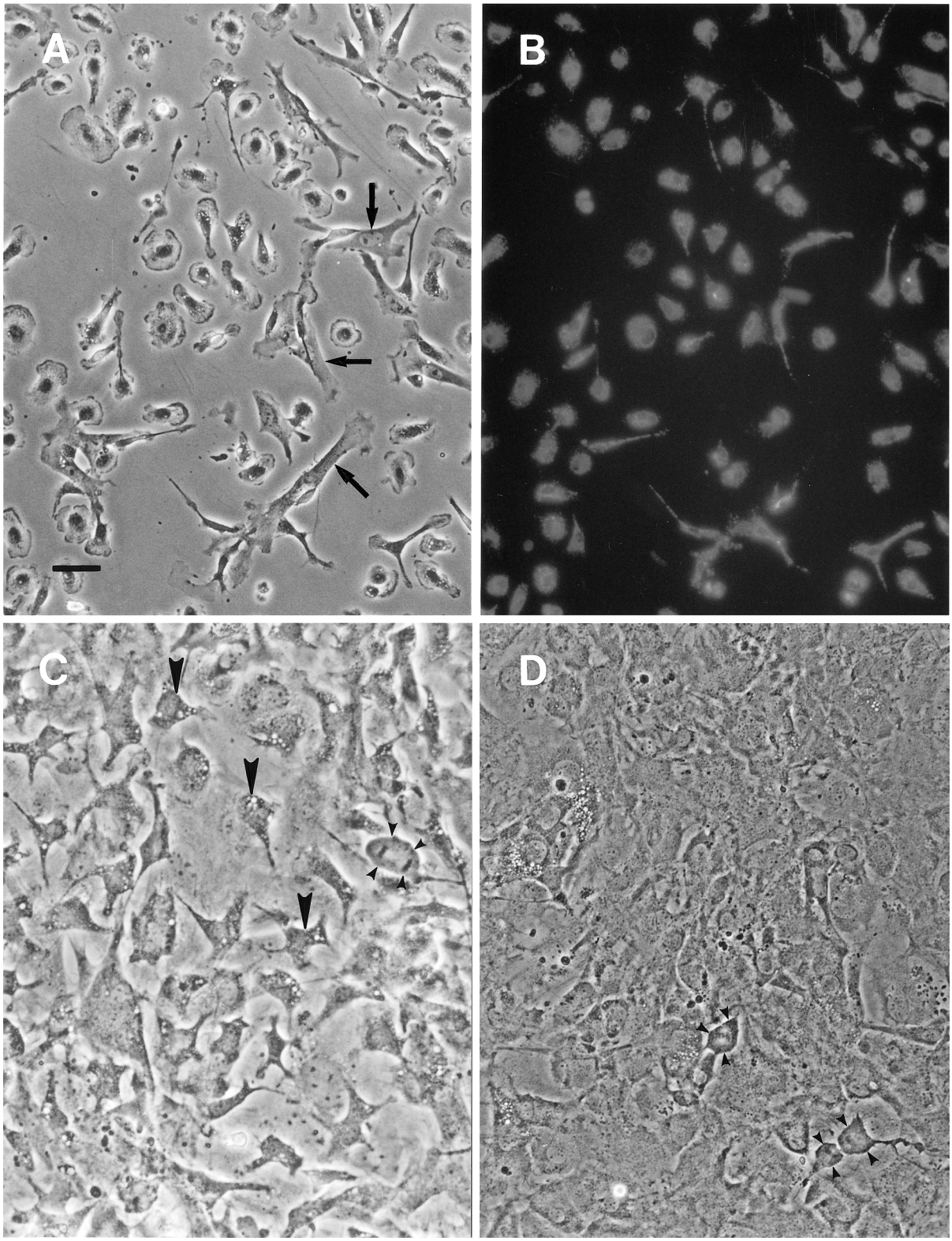

FIG. 1. Phase-contrast and fluorescence micrographs of mouse embryonic fibroblast–macrophage (MEF/Mac) cells in culture. (*A*) and (*B*) Phase-contrast and fluorescent microscopic comparison of the same area of interleukin-6 (−/−) macrophages (P5) treated for 4 h with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate–acetylated low-density lipoprotein (DiI-Ac-LDL), differentially attached and subsequently maintained in serum-free culture for 72 h; *note* DiI fluorescence in macrophages and nonfluorescence (no uptake of DiI-Ac-LDL) of several fibroblasts within the population (*arrows*). (*C*) C57BL/6 MEF/Mac culture (P4), where macrophages are seen as phase-contrast dark, flat cells with multiple pseudopodia (*large arrowheads*) that are closely associated with the underlying lighter fibroblasts. *Note* dividing cell (*small arrowheads*). (*D*) Passage 13 C57BL/6 MEF/Mac culture showing lack of macrophages within the monolayer (*small arrowheads* indicate two metaphase-stage fibroblasts within the culture). *Bar* = 39 μm for *A, B, D* and 26 μm for *C*.

low-glucose culture conditions, and their differential growth became obvious in the MEF/Mac cultures by 7–8 d postpassage. As the primary culture reached greater density (3–4 × 10^6 cells/T25 flask) during the 1-wk period, some of the macrophages became detached and floated in suspension in the culture. Besides the macrophages and MEF, no other cell types were obvious in the secondary cultures by microscopic examination.

Given the above observations, the differential growth of the MEF and macrophages was enumerated over extended secondary passage. Duplicate T25 flask cultures of C57BL/6, GCSF (−/−), or IL-6 (−/−) MEF/Mac were started in 10% DMEM/H medium by plating the cells from frozen aliquots of MEF/Mac cells (*see* Materials and Methods). When the cells in each flask reached confluency (48–72 h postplating), the cultures were pretreated with DiI-Ac-LDL to differentially label the macrophages, and cell counts were performed by hemocytometer to determine the total number of cells and the total number of DiI-Ac-LDL–positive cells (Fig. 2). In the first 48–72 h, the initial C57BL/6, GCSF (−/−), or IL-6 (−/−) cultures were composed of 35, 4.5, and 17.7% macrophages (DiI-Ac-LDL–positive cells), respectively. The remaining flask of each of the cultures was refed with 10% DMEM/L and cultured for 4 d in the low-glucose medium. The cultures were then treated with trypsin–EDTA to release the cells, which were then suspended in 10% DMEM/H medium for passaging into T12.5 flasks at a 1:4 split ratio. One of the T12.5 flasks of each cell culture was treated with DiI-Ac-LDL approximately 16 h after plating, and cell counts were performed to ascertain the number of macrophages and total cells at this time. The second-passage C57BL/6, GCSF (−/−), or IL-6 (−/−) cultures were composed of 31.7, 24.6, and 57.1% macrophage, respectively, after 16 h of culture in 10% DMEM/H. On the third d after passage, the remaining stock flasks were again refed with 10% DMEM/L and were again cultured for an additional 4 d before being passaged back into 10% DMEM/H on the seventh d. Another T12.5 flask of each second-passage 7-d culture was used to assay the number of DiI-Ac-LDL–positive cells in the culture. The second-passage 7-d C57BL/6, GCSF (−/−), or IL-6 (−/−) cultures were composed of 44.9, 27.6, and 62.5% macrophages, respectively (Fig. 2).

This culture regimen (3 d on 10% DMEM/H followed by 4 d on 10% DMEM/L) was maintained until the macrophages of each culture were no longer appreciably replicating (no net increase for a 1-wk culture period). Secondary MEF/Mac cultures were routinely passaged at a 1:4 split ratio at 7-d intervals. The C57BL/6, GCSF (−/−), or IL-6 (−/−) cultures were cultured for a total of 13, 14, and >32 passages, respectively (Fig. 2). Total cells in the initial cultures ranged from 5.8 × 10^6 cells/T12.5 flask to 10.6 × 10^6 cells/T12.5 flask, but then maintained at approximately 3 × 10^6 total cells/T12.5 flask until passage 9, and from that point gradually declined to ≤1 × 10^6 total cells/T12.5 flask (Fig. 2). The DiI-Ac-LDL–positive cell density declined at similar rates in all the cultures. The passage history of the macrophages (DiI-Ac-LDL–positive cells) in the MEF/Mac cultures showed a maintenance of cell densities of 1 × 10^6 to 2 × 10^6 DiI-Ac-LDL–positive cells/T12.5 flask during the first few passages or wk of secondary culture (Fig. 2). Thereafter, macrophage content gradually declined in all cultures. At 80–90 d in culture, corresponding to P10–11, the majority of the DiI-Ac-LDL–positive cells in the cultures were probably not replicating or doing so very slowly (Fig. 2). By P12 and P14, DiI-Ac-LDL–positive cells of the C57BL/6 and GCSF (−/−) cultures

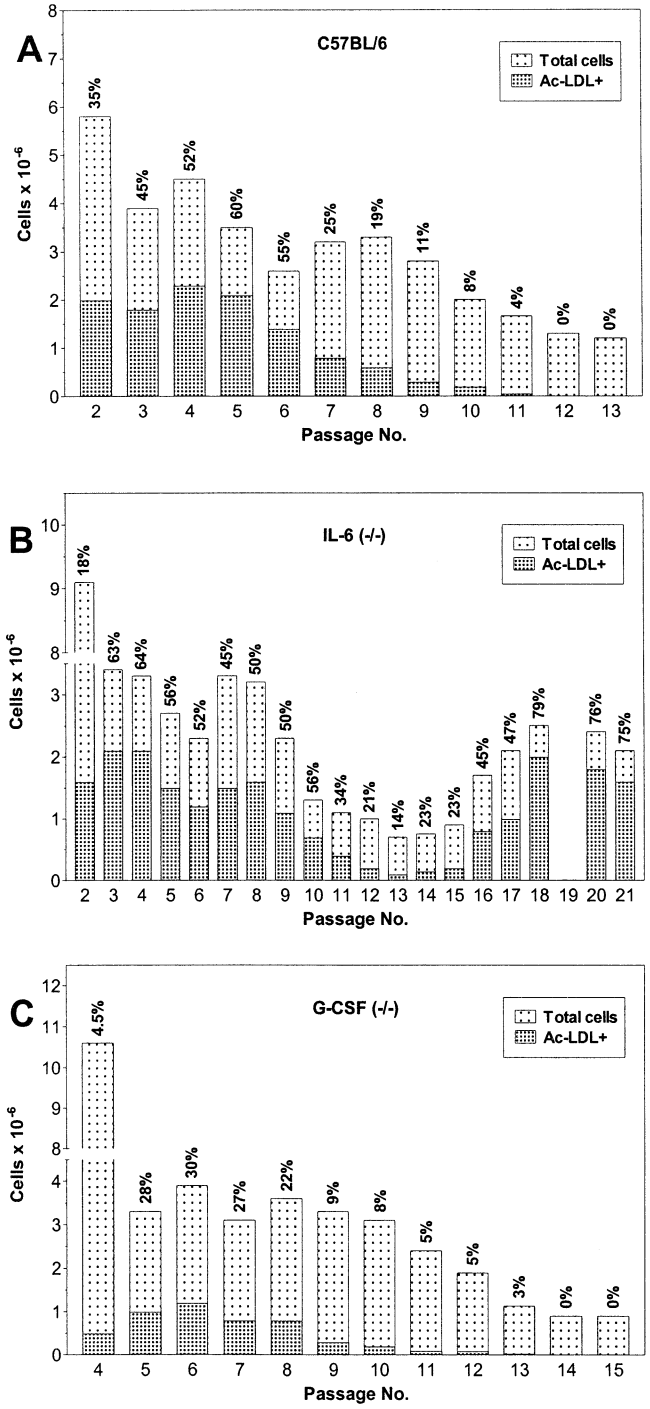

FIG. 2. Growth record of C57BL/6, interleukin-6 (−/−), and granulocyte colony stimulating factor (−/−) mouse embryonic fibroblast–macrophage cell cultures. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate–acetylated low-density lipoprotein (DiI-Ac-LDL)–positive cells compared with total cells per T12.5 tissue culture flask assayed 7 d postpassage are shown at each passage level (percentage of DiI-Ac-LDL–positive cells within the cell population is displayed at the *top* of each *bar*). (*A*) C57BL/6 culture. (*B*) Interleukin-6 (−/−) culture. (*C*) Granulocyte colony stimulating factor (−/−) culture. Each value is the mean of four hemocytometer counts.

fell to such a small percentage of the population that they were no longer found in the hemocytometer count areas, and were, therefore, counted as zero (Fig. 2*A* and *C*). Also, the macrophages were no longer visible among the fibroblastic cells by microscopic inspection (Fig. 1*D*). In contrast, the percentage of DiI-Ac-LDL–positive cells of the IL-6 (−/−) culture reached its lowest point (13.6%) at P13 and from there steadily rebounded until reaching a plateau percentage of approximately 75% at P21 and P22 (Fig. 2*B*). The IL-6 (−/−) cell culture, designated IL6MAC, was presumed to have spontaneously immortalized and was further cultured to passage 32 at which point it was composed of 45% macrophages and a total of 3.6×10^6 cells/T12.5 flask. The cumulative totals of macrophages (DiI-Ac-LDL–positive cells) produced for the C57BL/6 and GCSF (−/−) T12.5 flask cultures over their passage history were 11.5×10^6 cells and 5.0×10^6 cells, respectively. The IL-6 (−/−) culture produced the most robust outgrowths up to passage 13 with 14.1×10^6 DiI-Ac-LDL–positive cells in total. However, this cumulative tally was complicated by the IL-6 (−/−) culture's apparent spontaneous immortalization after passage 13 and its progression to stable yields of 1.5×10^6 to 2×10^6 macrophages/T12.5 flask (growth after 1 wk) at each passage to P32.

Culturing continuously in alternating high to low 10% DMEM was compared with culturing continuously in 10% DMEM/H for the percentage of macrophages produced and the total cells within a culture. At P5, the C57BL/6 culture had 59.6 versus 34.3% DiI-Ac-LDL–positive cells, respectively, and the IL-6 (−/−) culture had 55.9 versus 47.5% DiI-Ac-LDL–positive cells, respectively, for the two culture conditions tested (Fig. 3) Thus, the high–low glucose regimen produced a 1.74- and 1.17-fold increase in the percentage of macrophages within the C57BL/6 and IL-6 (−/−) cultures, respectively. Total cells per T12.5 flask were comparable between the two culture methods; 3.5×10^6 versus 3.1×10^6, respectively, for the C57BL/6 culture and 2.7×10^6 versus 2.4×10^6, respectively, for the IL-6 (−/−) culture at P5 (Fig. 3). Similar differences were observed at P6 (Fig. 3).

Primary or early secondary cultures from two other strains of mice (CF-1 and CD-1) were tested for macrophage content and propagation under the same conditions of cycling through 3 d in high glucose and 4 d in low glucose (Fig. 4). Concurrent cultures propagated in 10% DMEM/H at all times were also performed (Fig. 4). Compared with the C57BL/6 cell culture, the CD-1 and CF-1 cultures showed a similar content of macrophages after the first wk of culture with 42.6 and 39.6% DiI-Ac-LDL–positive cells, respectively. Similarly, the CD-1 and CF-1 cultures quickly reached a peak macrophage percentage of over 60% DiI-Ac-LDL–positive cells by P7 and P2, respectively. Also similarly, the percentage of macrophages steadily declined in the CD-1 and CF-1 cultures over subsequent passages. Both cell cultures, particularly the CF-1 culture, were notable in having relatively depressed total numbers of macrophages and fibroblasts in comparison with the C57BL/6 culture (Figs. 2 and 4). Continuous high–low glucose culture compared with continuous high-glucose culture produced a modest 1.16-fold gain in the percentage of macrophages for the CD-1 culture (64.9 versus 55.8% at P7) but a 2.1-fold increase in the percentage for

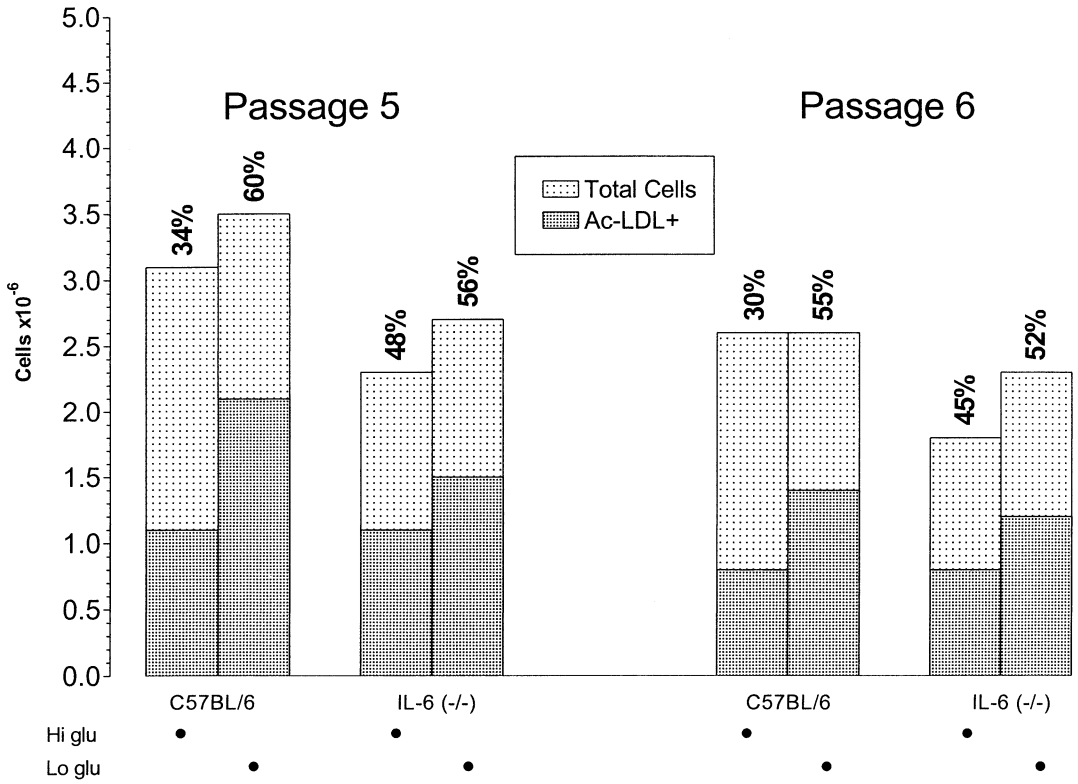

FIG. 3. Comparison of macrophage content and total cell content in C57BL/6 and interleukin-6 (−/−) cultures (P5 and P6) propagated in either Dulbecco modified Eagle medium (DMEM) with 4.5 g/L glucose (high glucose) supplemented with 10% fetal bovine serum (FBS), 2 m*M* glutamine, and 50 U/ml penicillin–streptomycin (10% DMEM/H) or alternating 10% DMEM/H followed by DMEM containing 1 g/L glucose (low glucose) and supplemented with 10% FBS, 2 m*M* glutamine, and 50 U/ml penicillin–streptomycin.

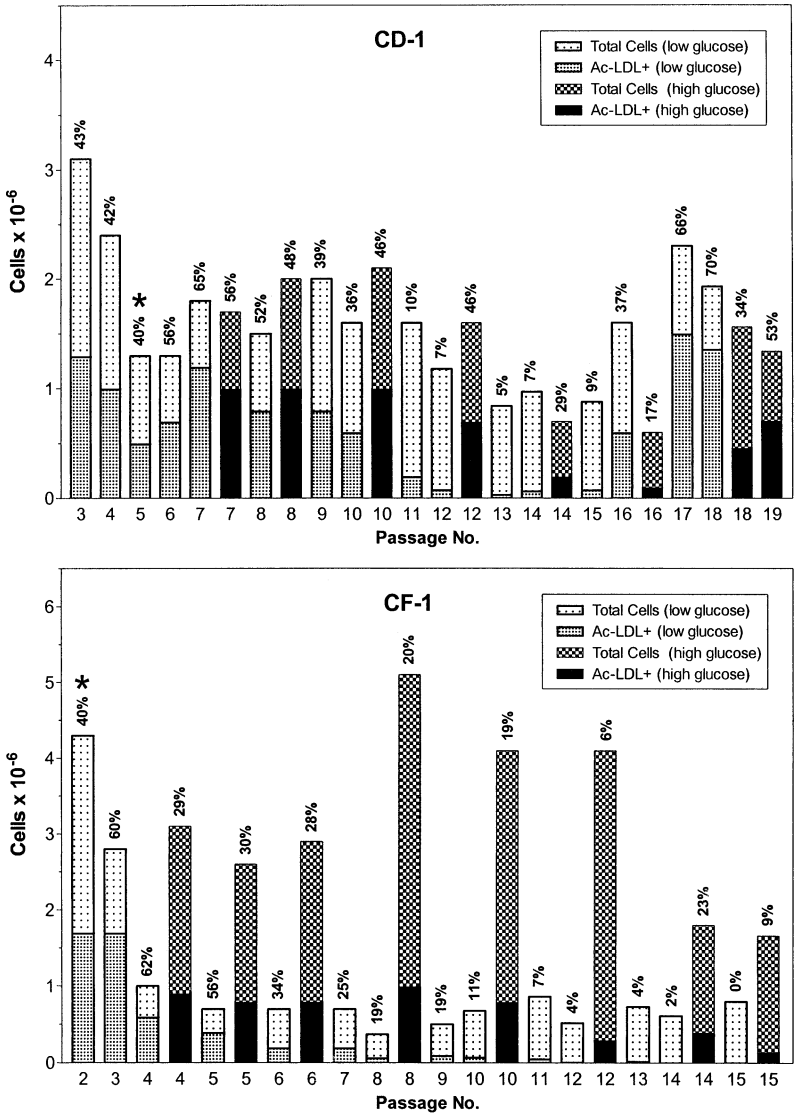

FIG. 4. Growth record of CD-1 and CF-1 mouse embryonic fibroblast–macrophage cell cultures. Data presentation is as for Fig. 2, and the *asterisk* indicates a single assay that was performed at 6 d postpassage instead of the usual 7-d postpassage time point. At several passage levels, cell counts for continuous Dulbecco modified Eagle medium (DMEM) with 4.5 g/L glucose (high glucose) supplemented with 10% fetal bovine serum (FBS), 2 m*M* glutamine, and 50 U/ml penicillin–streptomycin (10% DMEM/H) culture is presented side by side to the cell counts on the alternating 10% DMEM/H and DMEM containing 1 g/L glucose (low glucose) and supplemented with 10% FBS, 2 m*M* glutamine, and 50 U/ml penicillin–streptomycin culture regimen. Each value is the mean of four hemocytometer counts.

the CF-1 cultures (62.4 versus 29.4% at P4). However, whereas the total number of cells per T12.5 flask in the CD-1 cultures was comparable (1.8×10^6 versus 1.7×10^6), the total cell number in the continuous high-glucose culture was three times higher than the high–low glucose culture in the CF-1 cultures (3.1×10^6 versus 0.93×10^6, respectively). The CF-1 high–low glucose culture reached 0.0% macrophages at P15, at which point the total cells per T12.5 flask was only 8×10^5 cells. The high glucose only CF-1 culture had not reached 0% macrophages at this passage, but the culture was following this trend with only 9% macrophages and 1.7×10^6 cells total per flask. Similar to the IL-6 (−/−) culture, the CD-1 cultures (high–low glucose and high glucose only) both appeared to be spontaneously immortalized by the end of the assay period. The high–low glucose CD-1 culture was designated CD1MAC and had 70% macrophages within a total of 1.93×10^6 cells at P18. The high glucose only CD-1 culture was last assayed at P19 where it was composed of 53% macrophages with a total of 1.34×10^6 cells/T12.5 flask, and, therefore, also appeared to be spontaneously immortalizing.

Several analyses were performed on the MEF/Mac cultures to confirm that the phase-contrast dark, vacuolated, multipseudopod-bearing, and DiI-Ac-LDL–positive cells had other typical macrophage characteristics. The MEF/Mac cells had phagocytic activity (Table 1). After a 1-h exposure to FITC-labeled *S. aureus*, more than 85–90% of the MEF/Mac cells had bacteria adhering to them, and, after quenching the signal from external bacteria with methylene blue, most (80–70%) appeared to be internalized bacteria (Table 1). This very high percentage of cells exhibiting phagocytic

TABLE 1

PHAGOCYTIC ACTIVITY OF MEF/MAC CELL CULTURES[a]

	Cell associated percent fluorescence[b]	LMFC	Phagocytosed percent fluorescence	LMFC
C57BL/6[c]	86.5	215	70.5	34
IL-6 (−/−)[c]	85	147.5	72	32
G CSF (−/−)[d]	90.5	199	80.5	43

[a] Abbreviations: MEF/Mac, mouse embryonic fibroblast-macrophage; LMFC, log mean fluorescent channel, a measure of fluorescence intensity; IL-6, interleukin-6; GCSF, granulocyte colony for stimulating factor.
[b] Each value represents mean of duplicate determinations.
[c] Passage 5 in culture.
[d] Passage 6 in culture.

TABLE 2

ANTI-CD14 AND ANTI-CD25 MONOCLONAL ANTIBODY REACTIVITY

Cell type	Percentage cells fluorescing[a]	Log mean fluorescence channel[b]
C57BL/6		
CD14	66.9	16
CD25	7.8	3.8
IL-6 (−/−)[c]		
CD14	63	10.9
CD25	8.2	4
G CSF (−/−)[c]		
CD14	18.6	12.6
CD25	10.2	4.4

[a] Each value represents the mean of duplicate determinations and results are corrected for background.
[b] Log mean fluorescence channel is a measure of the fluorescence intensity and indicates receptor density. Cells were assayed at passage 5 and 6 as in Table 1.
[c] Abbreviations: IL-6, interleukin-6; GCSF, granulocyte colony stimulating factor.

activity was not consistent with the lower percentages of macrophages as assessed by DiI-Ac-LDL uptake (Fig. 2) and anti-CD14 antibody–reactive cells (*see* below). Transmission electron micrographs (TEM) of MEF/Mac cultures exposed to *S. aureus* bacteria explained this discrepancy because it was observed that bacteria were being phagocytized by both macrophages and fibroblasts (*see* below). Anti-CD14 monoclonal antibody was reactive with the C57BL/6, IL-6 (−/−), and GCSF (−/−) MEF/Mac, with 66.9, 63, and 18.6% of the cells being positive, respectively (Table 2). Alternatively, mouse-specific anti-CD25 that is reactive with T- and B cells gave relatively low reactivity with the MEF/Mac cells (Table 2).

The TEM analysis of an in situ 7-d C57BL/6 MEF/Mac culture was performed to observe the cell-to-cell organization of the monolayer. When cut in cross section, the in situ cells were found to be arranged in layers ranging from one to three cells thick (Fig. 5). Approximately equal numbers of macrophages and fibroblasts were present although the macrophages were usually situated underneath or above a central layer of fibroblasts. Morphologically, the two cell types were distinct. Macrophages were generally smaller and more compact or rounded in appearance and often had multiple pseudopods present at one or both ends of their horizontal aspect (Fig. 5). In general, the cytoplasm of the macrophages stained slightly darker than the fibroblasts' cytoplasm, and a macrophage's cytoplasm usually had one to several large "empty" or transparent vacuoles (Fig. 5). The nuclei of the macrophages were unremarkable but among the cytoplasmic organelles, Golgi complexes were particularly well represented along with rough endoplasmic reticulum (RER), mitochondria, and lysosomes (Fig. 6*A*). The fibroblastic cells were in contrast to the macrophages in being generally larger, being more spindle-like in shape, and having slightly lighter staining cytoplasm and an absence of well defined pseudopodia (Fig. 5). In addition, three other ultrastructure features were particularly characteristic of the fibroblastic cells. First, the cells were often filled with microfilament bundles, and arrays of bundles and similar microfilaments were emanating from and surrounding the fibroblasts (Fig. 6*B*). Second, the cells always contained numerous multivesicular bodies or digestive vacuoles of various sizes filled with complex mixtures of membrane-bound vesicles and unidentified material (Figs. 5 and 6*B*). A third distinguishing feature of the fibroblastic cells was their expanded RER (Figs. 5 and 6). Portions of the fibroblast's RER were sometimes expanded to the extent that it appeared as large or larger than the cell's nucleus. The nuclei of the fibroblastic cells were proportionately larger than those of the macrophages.

TEM analysis of suspended C57BL/6 and GCSF (−/−) MEF/Mac cells that were exposed to the FITC-labeled *S. aureus* was also performed. The C57BL/6 culture was approximately two-thirds macrophages as judged by their smaller size, their numerous and extended pseudopodia, their prominent Golgi complexes, and their generally oval nuclei (Fig. 8). The fibroblastic cells that were present were distinguished from the macrophages in that the fibroblasts were generally larger and had prominent expanded RER, large numbers of multivesicular bodies, and nuclei that were deeply enfolded in multiple places, which often gave them a "fractured" or tortuous outline in cross section (Fig. 7). Of several hundreds of macrophages observed over two sections, less than half of the macrophages had adherent or engulfed *S. aureus* associated with them, and the number of ingested *S. aureus* varied from a few to many dozens per cell (Fig. 8) This observation did not agree with the high phagocytosis rate as determined by flow cytometry (Table 1), although cells apparently negative for phagocytosis by TEM could easily contain bacteria because any single section through a cell represented a small portion of its three-dimensional content. However, most obviously, the discrepancy could be explained by the observation that many (~25%) of the fibroblastic cells had also engulfed bacteria or had bacteria associated with them (Fig. 7). Approximately 5% of the cells, mostly fibroblasts, in each grid examined were necrotic (Fig. 7), but no apoptotic cells were observed.

The TEM presentation of the GCSF (−/−) culture was similar in most respects to the C57BL/6 culture except for two prominent differences. First, the fibroblastic cells comprised greater than half of the cells in the sample, and the fibroblasts were often similar in size to the macrophages or only slightly larger. Second, more of the GCSF (−/−) fibroblasts appeared to have ingested *S. aureus* bacteria compared with the C57BL/6 fibroblasts, and the number of bacteria ingested per fibroblast could be relatively large, i.e., similar to that seen in some macrophages (not shown). This observation would explain the phagocytosis rate measured by flow cytometry, which was the highest in the GCSF (−/−) cells (Table 1). About

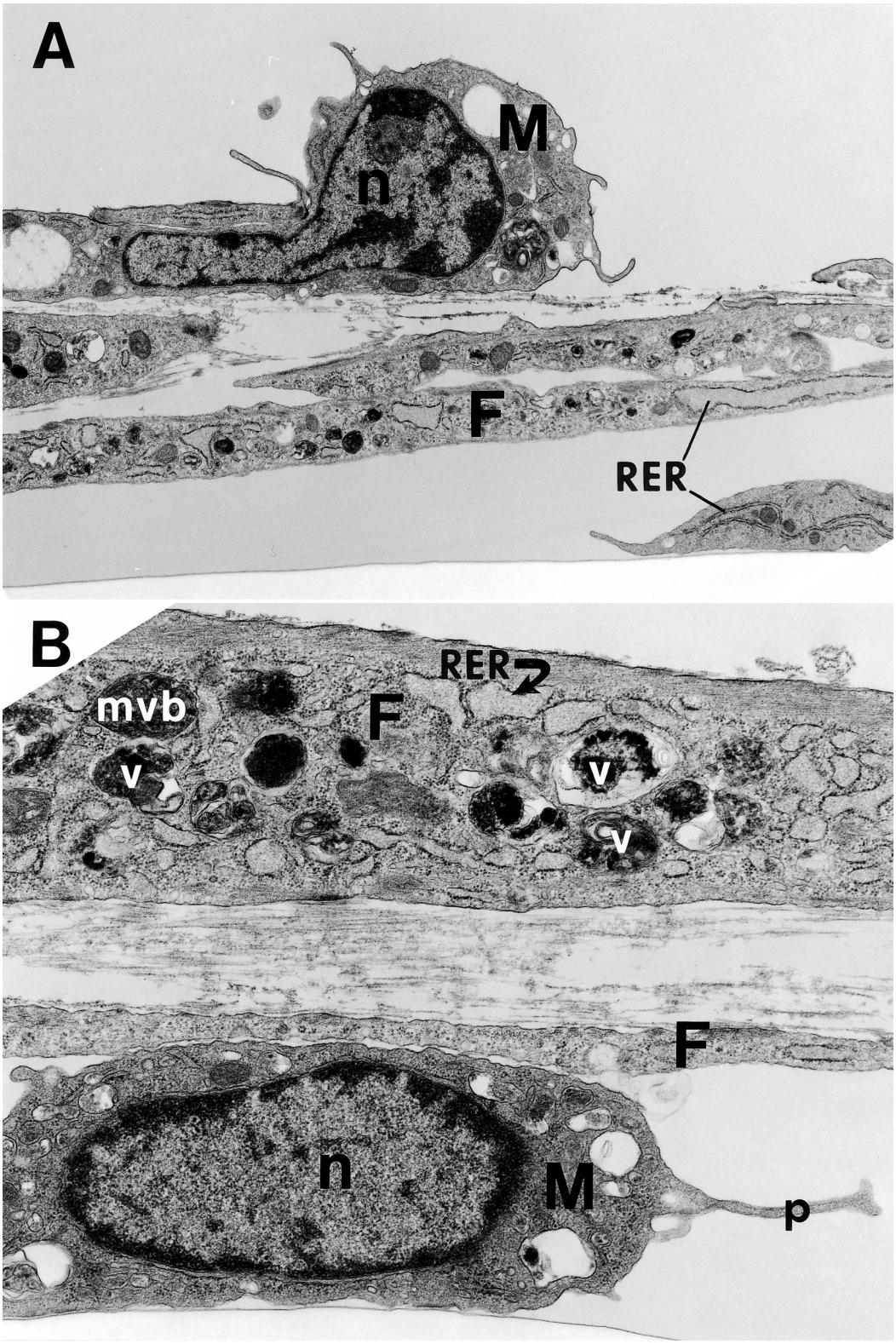

FIG. 5. Transmission electron micrograph of C57BL/6 mouse embryonic fibroblast–macrophage cells growing in situ on plasticware at P4. (*A*) Macrophages (M) are on top of the fibroblasts (F). *Note* expanded rough endoplasmic reticulum (RER) of the fibroblasts and the nonexpanded RER of part of a macrophage underneath the fibroblast at the plastic–resin interface. Magnification: ×9450. (*B*) Macrophages (M) are underneath the fibroblast (F) cell layer at the plastic–resin interface. *Note* the darker cytoplasm and pseudopod (p) of the macrophage. *Note* the multivesicular body (mvb), the numerous digestive vacuoles (v), and the expanded RER of the fibroblast (F). Nucleus (n). Magnification: ×24,000.

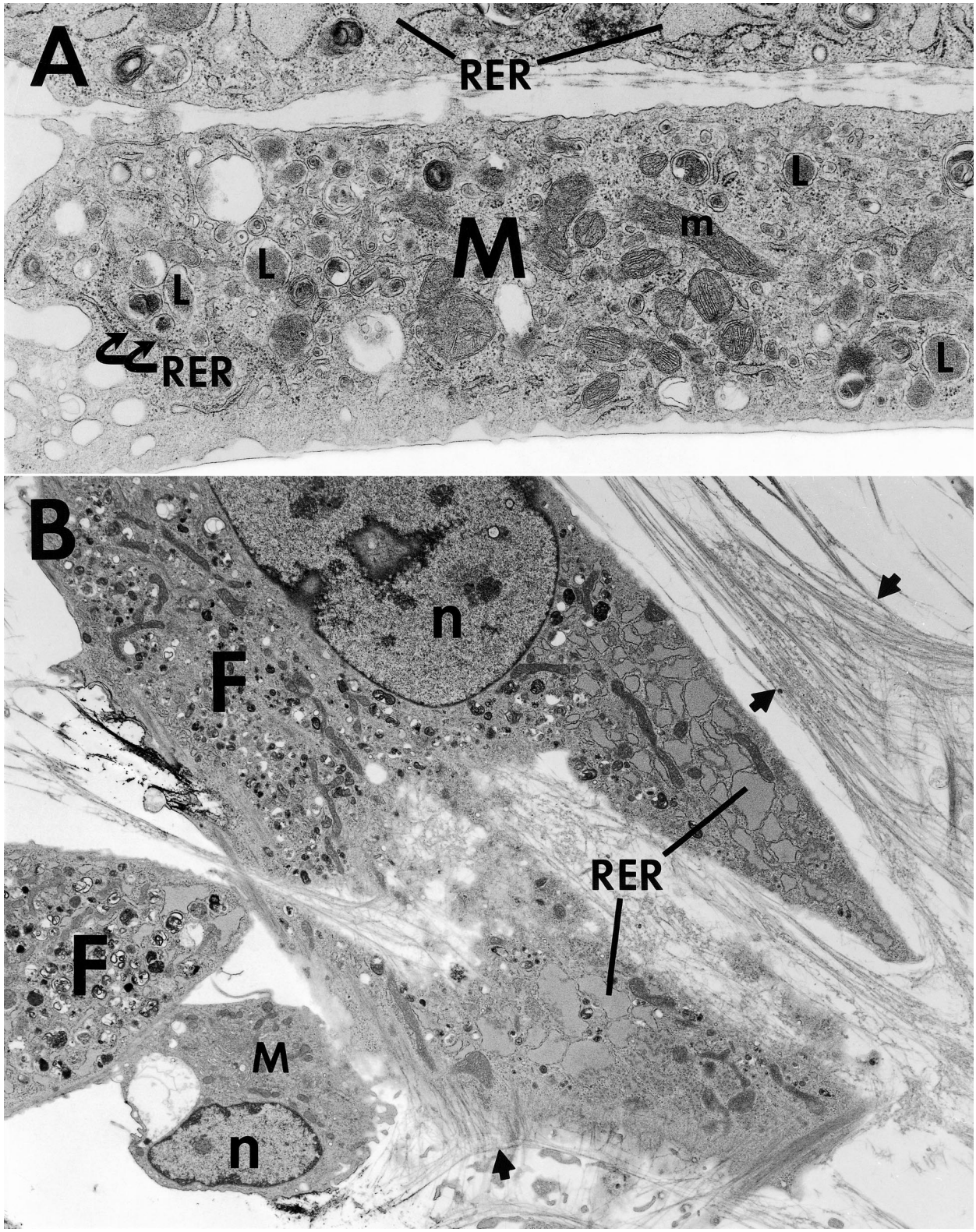

FIG. 6. Transmission electron micrograph of C57BL/6 mouse embryonic fibroblast–macrophage cells growing in situ on plasticware at P4. (*A*) Macrophages (M) at plastic–resin interface with fibroblast overtop. *Note* the numerous lysosomes (L) and the minimally expanded rough endoplasmic reticulum (RER) in the macrophages. The fibroblast has the characteristically greatly expanded RER. Mitochondria (m). Magnification: ×24,000. (*B*) In plane section of fibroblasts (F) and macrophages (M) showing the expanded RER of the fibroblasts, their associated extracellular fibers emanating from the fibroblasts (*arrows*), and their numerous multivesicular bodies and digestive vacuoles. Nucleus (n). Magnification: ×4800.

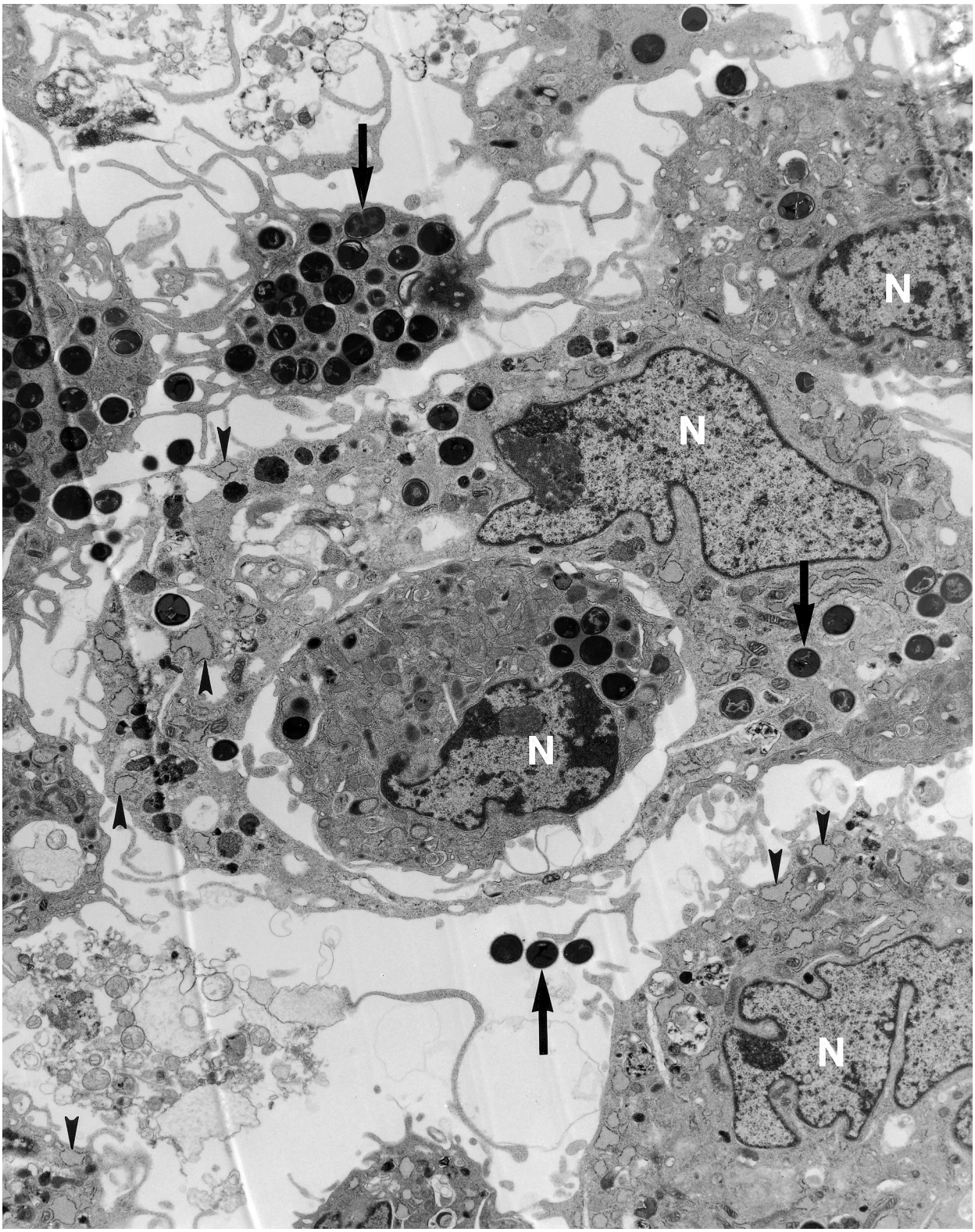

FIG. 7. Transmission electron microscopy photomicrograph of granulocyte colony stimulating factor (−/−) mouse embryonic fibroblast–macrophage cells (P6) after exposure to fluorescein isothiocyanate–labeled *Staphylococcus aureus*. *Staphylococcus aureus* bacteria (*arrows*) were phagocytized by the macrophages and the fibroblast cells. Fibroblasts were distinguished by their larger size, their having expanded rough endoplasmic reticulum (*arrowheads*), and by their deeply and multiply indent nuclei. In the center, *note* a macrophage containing ingested *S. aureus* being phagocytized by a larger fibroblastic cell also containing ingested *S. aureus*. N = nucleus. Magnification: ×7500.

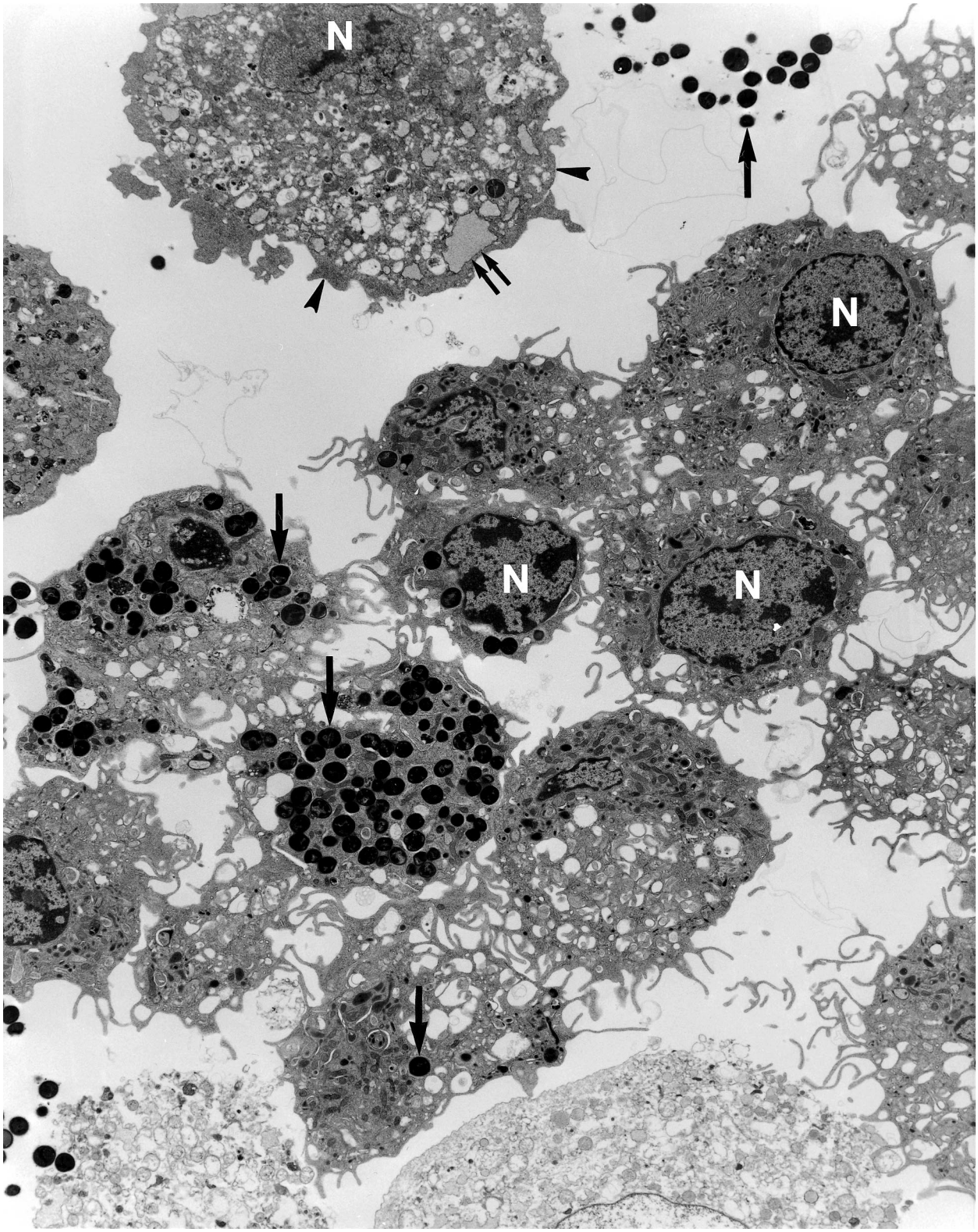

FIG. 8. Transmission electron microscopy photomicrograph of C57BL/6 mouse embryonic fibroblast–macrophage cells (P5) that have ingested multiple fluorescein isothiocyanate–labeled *Staphylococcus aureus*. Phagocytosis of *S. aureus* (*large single arrow*) bacteria is evident in several of the macrophages in the field. Although a fibroblast cell, indicated by *arrowheads* and identified by its expanded rough endoplasmic reticulum (*double arrows*) does not contain *S. aureus* bacteria in this particular field and section, other fibroblasts were found to contain multiple phagocytized bacteria. N = nucleus. Magnification: ×4800

1% of the fibroblastic cells were found to be ingesting their neighboring macrophages or fibroblasts in the GCSF (−/−) cell preparation (Fig. 7). This behavior was not unique to the GCSF (−/−) fibroblasts because one similar observation was made in a C57BL/6 section. Its occurrence was presumably more prevalent in the GCSF (−/−) cells because there were more fibroblastic cells in the GCSF (−/−) cell culture.

Fluorescent immunocytochemical staining with F4/80 antibody, specific for a 160-kDa cell surface glycoprotein present on macrophages, was undertaken to detect the cells in situ in a C57BL/6 MEF/Mac culture at P5. The antibody reacted with the phase-contrast dark, vacuolated, stellate cells that were also positive for DiI-Ac-LDL uptake (Fig. 9*A*). Macrophages that had adopted a semiattached morphology were also stained by the F4/80 antibody (Fig. 9*B*). The F4/80 cells appeared to comprise about 50% of the cells in the monolayer as judged by coincident Hoechst labeling of nuclei (not shown).

The cultured mouse macrophages could be purified from the fibroblastic cells by differential attachment. To demonstrate this, DiI-Ac-LDL–labeled macrophages were spun by centrifugation into a pellet so as to remove nearly all of the 10% DMEM medium that the cells were resuspended in after trypsin–EDTA treatment. The MEF/Mac mixture of cells was resuspended in serum-free DMEM, and the cells were plated on to a petri dish. The MEF/Mac cells were allowed to attach for 10 min, after which time 90–95% of the DiI-Ac-LDL–labeled macrophages were attached to the petri dish, and, in fact, mostly spread out flat on the plastic surface. The loosely adherent fibroblastic cells were easily dislodged by sharply banging the petri dish against one hand several times after removing most of the serum-free medium. The dislodged fibroblastic cells were removed by washing the plate with two to three changes of PBS. The cell culture, finally refed with 10% DMEM/H, was estimated to be composed of 95% macrophages by microscopic observation of DiI-Ac-LDL–labeled cells versus nonlabeled cells (Fig. 1*A* and *B*).

Discussion

Monocytes or macrophages are considered terminally differentiated cells normally capable of only limited in vivo and in vitro replication (Cohen and Cline, 1971; Freshney, 1994; Walker, 1994). However, the results of the present study show that normal mouse macrophages from various strains of mice can be effectively continuously cultured by coculture with resident fibroblastic cells. Also, some enrichment of the percentage of macrophages in the continuous cultures could be achieved by a regimen of cycling the cultures through high- and low-glucose DMEM medium exchanges. Even a strain of mouse carrying gene deletions, i.e., GCSF (−/−) that compromise in vivo macrophages formation and function (Lieschke et al., 1994), were shown to produce continuous cultures of macrophages.

The cumulative totals of macrophages (DiI-Ac-LDL–positive cells) produced were highest for the C57BL/6 mouse–derived and IL-6 knockout mouse–derived cultures at 11.5×10^6 and 14.1×10^6 macrophages, respectively. Cultures derived from the GCSF knockout, CD-1, and CF-1 mouse embryos were less productive with totals of 5×10^6, 7.1×10^6 (to P14) and 5.1×10^6 total macrophages produced, respectively, during the culture period. These totals were from culture in single T12.5 flasks and were therefore very small cultures compared with what could be expected to be routinely accomplished. For example, if all C57BL/6 cells from the original T12.5 flask had been cultured at each passage, an expected yield of 37×10^6 macrophages from a total area comprising two T75 flasks and two T25 flasks would result by the fourth passage. Because a single embryo can easily initiate a T75 flask primary culture, an estimated 896 million macrophages would be the expected harvest from the fourth passage of such a culture.

Although lowering the glucose level from 4.5 to 1.0 g/L in the culture system improved the differential growth of the macrophages, probably by inhibiting fibroblast growth, it was not essential across all mouse strains tested. Only two out of the four cultures tested had a marked improvement of macrophage content, the C57BL/6 and CF-1 cultures. And, even though the percentage of macrophages was 2.1-fold lower under continuous high-glucose culture conditions in the CF-1 culture, the actual yield of macrophages would be greater because the overall population density was 3-fold higher under high glucose. Thus, although lowering the glucose level did not markedly affect the total number of cells produced in three out of the four mouse strains compared, in the case of the CF-1 mouse strain, the overall poor growth resulting from lowering the glucose level produced a negative net effect in macrophage propagation. The differences in the response of the separate cultures indicate that optimal culture conditions for maximum production of macrophages from a particular strain of mouse may require empirical adjustments.

The fibroblastic cells within the MEF/Mac cultures may be acting as feeder cells by elaborating soluble factors that are mitogenic to their neighboring macrophage cells. For example, the conditioned medium from mouse lung fibroblasts mitotically stimulated mouse macrophages isolated by lung lavage (Naum, 1975). It has been shown that monocytes and macrophages can be mitotically stimulated by colony stimulating factor (CSF-1) and that, in contrast, granulocytes and lymphocytes did not respond and grow in the presence of CSF-1 (Tushinski et al., 1982; Genovesi et al., 1989; Walker, 1994). It is probable that CSF-1 is expressed by the fibroblastic cells coincidently cultured with the macrophages in the MEF/Mac cultures, and it may be a contributing factor in the macrophage replication experienced in the coculture (Stanley and Heard, 1977; Wu et al., 1990). Direct cell-to-cell interaction between the fibroblasts and macrophages that promote contact with membrane-bound factors and extracellular matrix components may also foster proliferation in the MEF/Mac cultures. As noted previously for epiblast-derived and fetal pig–derived macrophage cultures using feeder cells (Talbot and Paape, 1996), removing the mouse macrophages from the fibroblasts by differential plating resulted in a loss of macrophage growth promotion in that no apparent increase occurred in the number of macrophages as judged by microscopic observations over a subsequent 7–10 d of culture. Membrane-bound CSF-1 has been shown to stimulate monocytes or macrophages (Stein et al., 1990), and the necessity of intimate cell-to-cell contact for specific membrane-bound ligand interaction to promote growth responses has been demonstrated in various cell culture systems (Roberts et al., 1988; Matsui et al., 1992; Davis et al., 1994; Kobari et al., 1995).

The phagocytosis assay was confounded by the phagocytic activity of the fibroblasts within the culture. Although not shown, many fibroblasts were found to have ingested large numbers of the FITC-labeled *S. aureus* when examined under the electron microscope.

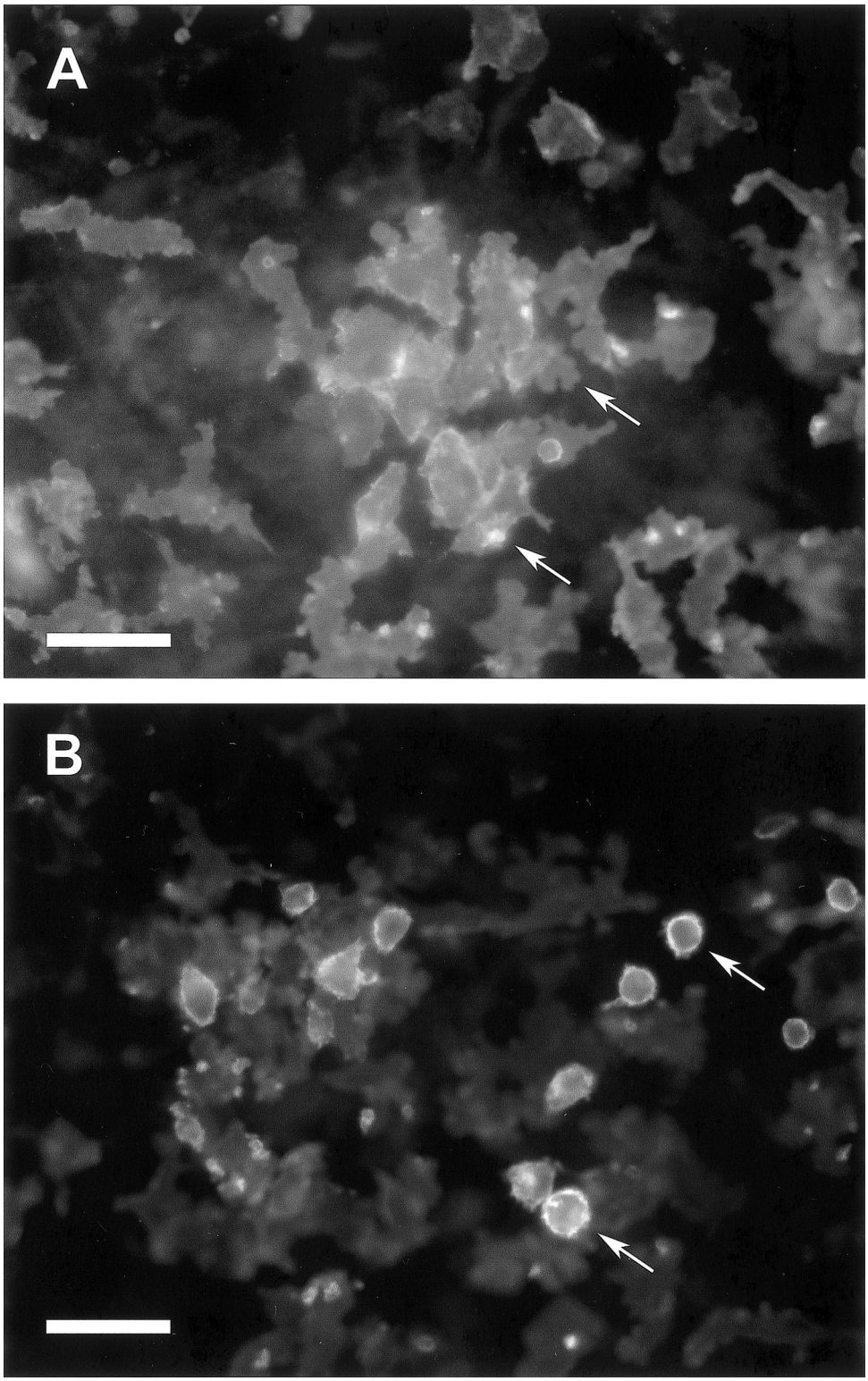

FIG. 9. F4/80 antigen expression in C57BL/6 macrophages at P5. (*A*) Positive reactivity of anti-F4/80 antibody with macrophages with flattened morphology. (*B*) Positive reactivity of anti-F4/80 antibody with macrophages with a rounded, semiattached morphology. *Bar* = 50 μm.

The TEM survey of over 100 cells gave no indication that the fibroblasts were any less phagocytic under the conditions of the assay than the macrophages. This conclusion was enabled by ultrastructure differences between the cells, most notably that the fibroblasts had numerous multivesicular bodies and digestive vacuoles and had RER that was prominently expanded. These TEM observations were reflected in flow cytometry data that showed a high percentage of cells with phagocytosed bacteria in comparison with a lower percentage of cells that were positive for CD14 and acetylated-LDL receptor (Fig. 2; Table 2). This was particularly evident, for example, in the GCSF (−/−) mouse–derived culture (at P6), where relatively low percentages of macrophages were found by light microscope observation, CD14 assay (18.6%; Table 2), and DiI-Ac-LDL uptake (26.6%; Fig. 2), but phagocytosis of FITC-labeled *S. aureus* was 80.5% (Table 1) for internalized bacteria.

Mouse and human embryonic stem (ES) cells are often maintained and grown on feeder layers made either from primary MEF cultures or early secondary MEF cultures. In fact, it is only recently that the feeder-independent growth of undifferentiated human ES cells has been demonstrated and this was dependent on medium conditioned by MEF (Xu et al., 2001). It was of interest, therefore, to examine CF-1 mouse–and CD-1 mouse–derived MEF cell cultures for macrophage content because feeder cells are often made from the fetuses of these mice (Ledermann and Bürki, 1991; Thomson et al., 1998). Macrophages, being producers of many secreted products, some with differentiation-inducing potential, might influence the quality of the feeder cells if they became a significant proportion of the population of cells comprising the MEF culture (Nathan, 1987; Rappolee and Werb, 1992). This study demonstrates that under common secondary culture conditions (i.e., 10% DMEM medium with 1:4 split ratios on plasticware), the macrophages within the cell population can reach 50–60% of the population over the first few passages of the culture. This was particularly true in medium with glucose levels of 1 g/L (a common amount in various media), where fibroblast growth was adversely affected. Laboratory practice in the isolation and maintenance of ES cells has often been disposed to the idea that feeder cells lose their ES cell supportive function after just a few passages in culture. The coincidence of this with the significant macrophage outgrowth in the early passages of primary MEF cultures might be causally connected because macrophages-derived growth factors can have negative effects on fibroblast growth (Bitterman et al., 1986; Gonzalez-Ramos et al., 1996) and ES cell growth (Kohchi et al., 1996; Wuu et al., 1998) or may stimulate the differentiation of ES cells (Gualandris et al., 2000; Kramer et al., 2000).

The GCSF-deficient MEF/Mac cultures (Lieschki et al., 1994) and cultures derived from IL-6 knockout mouse (Kopf et al., 1994) examined the in vitro growth of macrophages from immunodeficient mice that showed reduced numbers of macrophages in vivo or a reduction in macrophage-related immune functions, respectively. The approximate 50% reduction in circulating monocytes observed in 6-mo-old GCSF (−/−) mice (Lieschke et al., 1994) was recapitulated in vitro, where macrophage percentages in the GCSF (−/−) MEF/Mac cultures were about half of that experienced in C57BL/6, CF-1, and CD-1 MEF/Mac cultures. This may indicate that the culture system would model other strains of mice with more or less severely reduced in vivo monocyte or macrophage populations. The IL-6 deficient mice do not show consistent changes in their in vivo macrophage population (Bluethmann et al., 1994), although both positive and negative effects on macrophage tissue infiltration have been noted in these mice (Penkowa et al., 1999; Fisher et al., 2001). Otherwise, these mice have complex immunodeficient traits that appear to arise from accessory macrophage functions (Kopf et al., 1994; Dalrymple et al., 1995). Thus, perhaps it is not surprising that the MEF/Mac cultures produced similar percentages and numbers of macrophages as found in the C57BL/6 culture, its most closely matched control culture.

Of the five strains of mice used in the study, only the IL-6 knockout– and CD-1–derived cultures apparently immortalized to establish cell lines of continuously replicating macrophages (however, for the CD1MAC cell line, this conversion is more speculative because the cell culture was frozen at passage 19). Why these two cultures spontaneously immortalize and the GCSF (−/−), C56BL/6, and CF-1 cell strains did not is not known. Whether cultures derived from these mouse strains, or others, would or would not immortalize was not quantitatively examined in this study. Also, the effect of high- or low-glucose culture on immortalization was not examined; although (with n = 1) it appeared for the CD-1 cultures that immortalization (or recovery of growth potential) occurred under both the glucose conditions tested (Fig. 4). For mechanistic and genetic inquiry, it might be of interest to test whether the immortalizations were the result of random chance or whether certain mouse strains were indeed predisposed to MEF/Mac immortalization in culture.

In conclusion, the merits of this macrophage culture method are that macrophages from any strain of mouse can probably be grown and thus compared in vitro; standard explant culture technique, plasticware, and media are used; rapid expansion of the macrophage population occurs within two to three passages; macrophages are released by trypsin–EDTA treatment (i.e., the fibroblasts appear to condition the surface or the macrophages never adhere to the plastic); differential plating of the macrophages on petri dish plastic in serum-free medium is effective in producing purified macrophage cultures; and establishment of permanent cell lines may occur. The establishment of a macrophage cell line from the IL-6–deficient mouse could enable further analyses of the functional consequences of loss of IL-6 expression in macrophages and other cells that interact with macrophages. The correlation between the macrophage outgrowth presented in this study and the failure of early secondary mouse fibroblasts cultures as feeder cells for ES cell propagation, particularly human ES cells, may indicate a causal relationship. Finally, the data demonstrate that it should be appreciated that a substantial proportion of early secondary passage MEF cell cultures can be composed of macrophages.

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